

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 17 January 1997 (17.01.97)	
International application No. PCT/EP96/02747 ✓	Applicant's or agent's file reference A 2176 PCT
International filing date (day/month/year) 24 June 1996 (24.06.96)	Priority date (day/month/year) 23 June 1995 (23.06.95)
Applicant DICKMANNNS, Achim et al	

1. The designated Office is hereby notified of its election made:



in the demand filed with the International Preliminary Examining Authority on:

30 December 1996 (30.12.96)



in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<p>The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland</p> <p>Facsimile No.: (41-22) 740.14.35</p>	<p>Authorized officer Eugénia Santos</p> <p>Telephone No.: (41-22) 730.91.11</p>
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PATENT COOPERATION TREATY

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From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

VOSSIUS & PARTNER
Patentanwälte
Postfach 86 07 67
D-81634 München
ALLEMAGNE

EINGEGANGEN
Vossius & Partner GbR

24. Sep. 1997

Frist
beerb.:

NOTIFICATION OF TRANSMITTAL OF
INTERNATIONAL PRELIMINARY
EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing
(day/month/year)

22 SEP 1997

Applicant's or agent's file reference
A 2176 PCT

IMPORTANT NOTIFICATION

International application No.
PCT/EP 96/ 02747

International filing date (day/month/year)
24/06/1996

Priority date (day/month/year)
23/06/1995

Applicant

MICROMET GMBH et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/



European Patent Office
D-80298 Munich
Tel. (+49-89) 2399-0, Tx: 523656 epmu d
Fax: (+49-89) 2399-4465

Authorized officer

Peter Ehrenreich

Peter Ehrenreich

Telephone No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference A 2176 PCT	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP 96/02747	International filing date (day/month/year) 24/06/1996	Priority date (day/month/year) 23/06/1995
International Patent Classification (IPC) or national classification and IPC C12N5/10		
Applicant MICROMET GMBH et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.


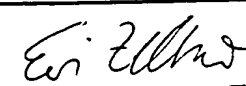
2. This **REPORT** consists of a total of 6 sheets, including this cover sheet.

☐ This report is also accompanied by **ANNEXES**, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of 4 sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 30/12/1996	Date of completion of this report 22 SEP 1997
Name and mailing address of the IPEA/  European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer  E. Zellner Telephone No. <u>8427</u>

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

I. Basis of the report

1. This report has been drawn up on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

☐ the international application as originally filed.

☒ the description, pages 1-33 _____, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____.

☒ the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. 1-28 _____, filed with the letter of 18.08.97,
Nos. _____, filed with the letter of _____.

☒ the drawings, sheets/fig 1/-5/5 _____, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☐ the description, pages _____.

☐ the claims, Nos. _____.

☐ the drawings, sheets/fig _____.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern. application No.

PCT/EP96/02747

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 16-22_____	YES
	Claims 1-4, 6-10, 24_____	NO
Inventive Step (IS)	Claims 16-22_____	YES
	Claims 1-15, 23-28_____	NO
Industrial Applicability (IA)	Claims 1-28_____	YES
	Claims _____	NO

2. CITATIONS AND EXPLANATIONS

1. The following documents (D) are referred to in this communication:

D1: Journal of the National Cancer Institute, 1989, 81(8), pp. 587-594

D2: Jnp. J. Cancer res. 1995, 86, pp. 347-356

D3: PNAS, 1991, 88, pp. 3520-3524

2. D1 describes normal bronchial epithelial (NHBE) cells which have been immortalized by transformation of the SV40 T antigen, thus obtaining BEAS-2B cells. BZR cells are generated by transfer of the vHa-ras oncogene. Said BZR cells are tumorigenic in an xenotransplantation model and formed carcinomas with lung metastasis (page 588, left column, paragraph 3 - paragraph 8, right column, paragraph 1 - 2).

Therefore the cells described in D1 are indistinguishable from the cells defined in Claims 1-4, 5-10 (Article 33 (2) PCT), since they are also immortalized, have integrated an externally introduced immortalizing oncogene

and are tumour cells with metastatic potential.

The fact that the cells of the present application have been produced in a manner different from the way described in D1 is not expressed in Claim 1 but only in the process claims 16-28.

The use of said cells in in vivo diagnosis is also cited in D1 (page 587, left column, last paragraph and page 591, right column, line 18 - page 592, line 1). Therefore Claim 24 is not novel.

3. For a skilled person a generic use of known artificially modified tumour cells in prophylaxis or treatment of cancer (Claims 23 and 25-28) appears to be obvious and thus is not considered as to involve an inventive step.

The feature that the epithelial cells can be derived from bone marrow instead of bronchial cells appears to be obvious for a skilled person and thus Claim 5 does not involve an inventive step.

4. The production of antibodies specifically recognizing known cells is not inventive since it merely implies routine techniques.
Therefore claims 13-15 are not allowable under Article 33 (3) PCT.

5. The feature of Claims 11 and 12 "one externally introduced gene encoding an immunostimulatory factor" is "optional" according the description (page 1, line 4). Therefore said feature is not essential in order to solve the problem of the alleged invention (Article 33 (3) PCT).

6. Process Claims 16-22 appear to be novel and inventive for the following reasons.
-

The essential feature distinguishing said claims from the closest prior art document D1 is that an epithelial tumour cell line is immortalized, whereas in D1 normal human bronchial cells are immortalized and then transformed into tumour cells.

The problem of the present invention is another suitable tool in cancer research (page 4, paragraph 2, page 12, paragraph 4) providing an unlimited expansion of tumour cells of epithelial origin with metastatic potential. The solution, that tumour cells are immortalized by an external immortalizing oncogene is not derivable from the prior art in an obvious manner.

In D2 non-metastatic epithelial tumour cells are obtained by irradiation and the metastatic potential is examined by introduction of oncogenes.

In D3 non-tumorigenic cells are immortalized with an external immortalizing oncogen such as the SV40 T antigen for further studying carcinogenesis. However, none of the prior art documents suggests to immortalize tumour cells by and additional external oncogen.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The wording "or" in Claim 1 is not clear within its context.

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MICROMET GMBH et al.
our ref.: A 2176 PCT

CLAIMS

1. An immortalized epithelial tumor cell with metastatic potential which has integrated in its genome or another replicative genetic element an externally introduced immortalizing oncogene which is expressed in said cell.
2. The epithelial tumor cell according to claim 1 which is a disseminated tumor cell.
3. The epithelial tumor cell according to claim 1 or 2 which is an autologous tumor cell.
4. The epithelial tumor cell according to any one of claims 1 to 3 which is a human tumor cell.
5. The epithelial tumor cell according to any one of claims 1 to 4 which is derived from bone marrow.
6. The epithelial tumor cell according to any one of claims 1 to 5, wherein said immortalizing oncogene is the DNA encoding the early region of SV40 DNA and preferably the large T antigen of a replication deficient SV40 virus.
7. The epithelial tumor cell according to claim 6, wherein said replication deficiency is caused by (a) defect(s) in the origin of replication and/or (a) defect(s) in the antigen coding region.
8. The epithelial tumor cell according to claim 6 or 7, wherein said SV40 virus is non-infectious.
9. The epithelial tumor cell according to any one of claims 1 to 8 which has integrated in its genome at least one additional oncogene.

AMENDED SHEET

10. The epithelial tumor cell according to claim 9, wherein said additional oncogene is ras, mutant WT1, bcl-2, p53mut, myc, HER 2/neu, an HPV16 oncogene, an HPV18 oncogene or E1A.
11. The epithelial tumor cell according to any one of claims 1 to 10, which has additionally integrated in its genome or another replicative genetic element at least one externally introduced gene encoding an immunostimulatory factor.
12. The epithelial tumor cell according to claim 11, wherein said immunostimulatory factor is B7 or a cytokine, preferably IL-2, IL-4, IL-7, IFN- α or IFN- γ .
13. An antibody or fragment thereof or a derivative of said antibody or said fragment thereof which specifically recognizes the tumor cell according to any one of claims 1 to 12.
14. The antibody or fragment thereof according to claim 13 which is monoclonal antibody or fragment thereof.
15. The derivative or fragment according to claim 13 which is a fusion protein or a chemical conjugate, preferably a bispecific antibody.
16. An in vitro process for the production of the tumor cell according to any one of claims 1 to 12 comprising the step of incorporating DNA comprising DNA encoding at least one immortalizing oncogene and optionally at least one gene encoding an immunostimulatory factor into a non-immortalized epithelial tumor cell with metastatic potential.
17. The process according to claim 16, wherein said step of incorporating DNA comprises microinjection or bombardment with DNA-coated microparticles.

18. The process according to claim 16 or 17, additionally comprising the step of carrying out a primary expansion of said non-immortalized epithelial tumor cells prior to the microinjection or bombardment step.
19. The process according to claim 18, wherein said primary expansion comprises the step of culturing tissue or a body fluid comprising non-immortalized epithelial tumor cells in a suitable medium promoting the expansion of said tumor cells.
20. The process according to claim 19, wherein said body fluid is bone marrow, blood, ascites or pleural exsudate.
21. The process according to claim 19 or 20, wherein said medium comprises EGF and/or bFGF, preferably rh EGF and rhb FGF.
22. The process according to any one of claims 19 to 21, wherein said culturing step is carried out in ECM-coated tissue flasks and/or at reduced oxygen concentrations of 5-10%.
23. A pharmaceutical composition comprising the epithelial tumor cell according to any one of claims 1 to 12 and/or the antibody or derivative or fragment thereof of any one of claims 13 to 15, optionally in combination with a suitable pharmaceutical carrier.
24. A diagnostic composition comprising the epithelial tumor cell according to any one of claims 1 to 12 and/or the antibody or derivative or fragment thereof of any one of claims 13 to 15.
25. Use of the epithelial tumor cell according to any one of claims 1 to 12 for the preparation of a medicament for the prophylaxis and/or treatment of cancer and/or metastasis of cancer.

26. Use of the antibody or derivative or fragment thereof according to any one of claims 13 to 15 for the preparation of a medicament for the prophylaxis and/or treatment of cancer and/or metastasis of cancer.
27. Use of the epithelial tumor cell according to any one of claims 1 to 12 or the antibody or derivative or fragment thereof of any one of claims 13 to 15 for the preparation of a tumor vaccine.
28. Use of the epithelial tumor cell according to any one of claims 1 to 12 for the ex vivo stimulation of a patient's immune cells.

AMENDED SHEET

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference A 2176 PCT	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/EP 96/ 02747	International filing date(<i>day/month/year</i>) 24/06/1996	(Earliest) Priority Date (<i>day/month/year</i>) 23/06/1995
Applicant MICROMET GMBH et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☐ Certain claims were found unsearchable (see Box I).
2. ☐ Unity of invention is lacking (see Box II).
3. ☒ The international application contains disclosure of a nucleotide and/or amino acid sequence listing and the international search was carried out on the basis of the sequence listing
 - ☐ filed with the international application.
 - ☒ furnished by the applicant separately from the international application,
 - ☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
 - ☐ Transcribed by this Authority
4. With regard to the title, ☒ the text is approved as submitted by the applicant.
 - ☐ the text has been established by this Authority to read as follows:
5. With regard to the abstract,
 - ☒ the text is approved as submitted by the applicant.
 - ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.
6. The figure of the drawings to be published with the abstract is:
 - Figure No. _____ ☐ as suggested by the applicant.
 - ☐ because the applicant failed to suggest a figure.
 - ☐ because this figure better characterizes the invention.
 - ☒ None of the figures.

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 5/10, C07K 16/30, A61K 39/00, 39/395, G01N 33/53		A1	(11) International Publication Number: WO 97/00946 (43) International Publication Date: 9 January 1997 (09.01.97)
(21) International Application Number: PCT/EP96/02747 (22) International Filing Date: 24 June 1996 (24.06.96) (30) Priority Data: 23 Dec 97/30 Nov 95 95109860.7 23 June 1995 (23.06.95) EP (34) Countries for which the regional or international application was filed: DE et al.		(81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(71) Applicant (for all designated States except US): MICROMET GMBH [DE/DE]; Am Klopferspitz 19, D-82152 Planegg (DE). (72) Inventors; and (75) Inventors/Applicants (for US only): DICKMANN, Achim [DE/DE]; Eichenstrasse 13, D-85649 Hofolding (DE); FAN-NING, Ellen [US/US]; Vanderbilt University, Dept. of Molecular Biology, P.O. Box 1820 STA B, Nashville, TN 37235 (US); PANTEL, Klaus [DE/DE]; Herderstrasse 22, D-85386 Echting (DE); RIETHMÜLLER, Gerhard [DE/DE]; Hermann-Vogel-Strasse 34, D-80805 München (DE). (74) Agent: VOSSIUS & PARTNER; P.O. Box 86 07 67, D-81634 Munich (DE).		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: IMMORTALIZED EPITHELIAL TUMOR CELL			
(57) Abstract <p>The present invention relates to epithelial tumor cells with metastatic potential which have integrated in their genome or another replicative genetic element at least one externally introduced immortalizing oncogene and optionally at least one gene encoding an immunostimulatory factor which are expressed in such tumor cells. The invention further relates to antibodies which specifically recognize the epithelial tumor cells of the invention, to processes for the production of said tumor cells as well as pharmaceutical and diagnostic compositions comprising said tumor cells and antibodies, respectively. Finally the present invention relates to the use of the epithelial tumor cells and/or antibodies of the invention for the preparation of tumor vaccines and medicaments for the prophylaxis and/or treatment of cancer and/or the metastasis of cancer.</p>			

Immortalized epithelial tumor cell

The present invention relates to epithelial tumor cells with metastatic potential which have integrated in their genome or another replicative genetic element at least one externally introduced immortalizing oncogene and optionally at least one gene encoding an immunostimulatory factor which are expressed in such tumor cells. The invention further relates to antibodies which specifically recognize the epithelial tumor cells of the invention, to processes for the production of said tumor cells as well as pharmaceutical and diagnostic compositions comprising said tumor cells and antibodies, respectively. Finally the present invention relates to the use of the epithelial tumor cells and/or antibodies of the invention for the preparation of tumor vaccines and medicaments for the prophylaxis and/or treatment of cancer and/or the metastasis of cancer.

Malignant tumors of epithelial tissues represent the majority of all new cases of cancer and fatalities due to cancer in the industrially developed countries of the Western world such as Germany. Due to the advancement in the early diagnosis of cancer the percentage of patients with an operable primary tumor has constantly risen during recent years. As a consequence the cancer mortality rate is increasingly determined by the early primary diagnosis of cancer at the time of which tumor cell dissemination is not yet apparent (Early breast cancer trialists' collaborative group, The Lancet 339:1, 1992; Frost and Levin, The Lancet 339:1458, 1992). Since so far single, disseminated tumor cells can neither be demonstrated in tissue using high-resolution imaging diagnostic procedures nor with conventional histopathological tests, (Schlimok et al., Proc. Natl. Acad. Sci. USA 84:8672, 1987), the prognosis

factors considered for a therapy decision for the patient only depends on statistical indices of the prognosis. Systemic adjuvant therapies applied after the surgical resection of the primary tumor for the secondary prevention of metastatic relapse are often accompanied by serious toxic side-effects (Hillner and Smith, New Engl. J. Med. 324:160, 1991; Early breast cancer trialists' collaborative group, loc. cit.).

Therefore, a need in the art exists to identify epithelial tumor cells prone to become the nucleus of a metastasis at a stage of tumor development as early as possible. Additionally, it would be highly advantageous to provide an experimental system which is suitable for the identification of such tumor cells after dissemination from the primary tumor and infiltration of a secondary tissue. The identification of said tumor cells could then be used to develop detection means for said cells as well as pharmaceutical compositions which may be employed in the prophylaxis or treatment of metastasis.

So far the successful development of the desired methods and tumor cells, respectively, has been hampered by a variety of factors. One of said factors is the development of suitable antibodies which specifically recognize epithelial tumor cells with metastatic potential. Another problem is that prior to the formation of metastasis, these tumor cells can be found only in very low percentages in the infiltrated tissue such as bone marrow, said percentages being in the range of 10^{-5} to 10^{-6} . Since these cells do not divide but rest in the infiltrated tissue, conventional tumor therapies which rely on cell proliferation, fail with the eradication of these cells. Moreover, a different expression of surface markers as compared to primary tumor cells allows the epithelial tumor cells with metastatic potential in general to evade from antibody based diagnosis or therapy.

Progress has in recent years mainly been made with the conventional histopathological methodology which has proved to be useful in the demonstration of micrometastatic tumor cell aggregates (Burkhardt et al., Bull. Cancer 67:2, 1980; Grunow and Groetchen, Pathologe 12:210, 1991). These methods have, however, failed in the detection of single epithelial tumor cells with metastatic potential.

Another approach for the detection of epithelial tumor cells with metastatic potential is the employment of molecular biology tools. Thus, the system developed by Kahn et al., Oncogene 6:1079, 1991, combining PCR amplification and subsequent restriction digestion of the amplified product can be used to detect mutations in codon 12 of the Ki-ras oncogene of micrometastasing cells. However, such a process is relatively time consuming and does not provide any living cell material for further study or a development of cell cultures useful for the preparation of diagnostic or pharmaceutical compositions or for the generation of antibodies.

An additional possibility of approaching the above outlined problem is to transform the desired cells and thus generate continuous growing cell lines. However, methods such as retroviral gene transfer are, due to the low number of these cells in primary tumors as well as in infiltrated tissue, only of theoretical value. The method described in EP-B1 0 256 512 suffers from the disadvantage that, applied to the present problem, an unspecific transformation of culture cells occurs, i.e. that not only the actual target cell, but a large number of other cells is immortalized as well. Further, the use of not defined transforming DNA precludes the use of immortalized cells for pharmaceutical purposes.

Finally, DE-PS 44 22 570 discloses a method of transforming stromal cells with a replication deficient SV40 virus. Whereas this result might have been expected, since the

transforming principle of SV40, the large T antigen, is known to interact (and inactivate or interfere with) the tumor suppressor gene p53 which in turn controls the transition of the G0 into the G1 cell cycle phase, the person skilled in the art would not expect any corresponding result in the case of tumor cells (Levine et al., Brit. J. Cancer, 69:409, 1994, Sussi et al., In. J. Cancer, 57:1, 1994). This is because the p53 protein is known to be frequently defective or absent in epithelial tumor cells (Harris and Hollstein, N. Engl. J. Med. 329:1318, 1993) and still do these cells grow even worse in culture than normal adherent cells. An interaction of the SV40 large T antigen would therefore not, according to all reasonable explanations, lead to a proliferative response of immortalized tumor cells.

In view of the above recited difficulties the technical problem underlying the present invention was to provide an epithelial tumor cell with metastatic potential that can be used for the study and development of compounds or compositions of diagnostic and therapeutic value.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to an epithelial tumor cell with metastatic potential which has integrated in its genome or another replicative element an externally introduced immortalizing oncogene which is expressed in said cell.

The tumor cell of the present invention may be used to generate large quantities of cells that are derived from the earliest metastasizing cells and that apparently have conserved the phenotype of the residual tumor cells present in the patient. The availability of such cells opens a new avenue to an in-depth molecular analysis of cancer

micrometastasis and may, e.g., be useful as a novel source for autologous tumor cell vaccines. The great advantage of this source is that it could be applied in the critical stage of minimal residual cancer, when the tumor load is minimal and the immune system is still intact.

The term "metastatic potential" describes the potential of said epithelial tumor cell to be the nucleus of metastatic formation. It is known that the formation of tumor cells is a complex and multistage process. The first step is the generation of cell variants in the primary tumor which subsequently detach from the tumor coenobium and proceed after invasion and penetration of the stromal tissue to the lymph or blood vessel system (Hart and Saimi, *The Lancet* 339:1453, 1992). As is known from early work in this field, cell clones already develop in the early growth phase of a tumor which display significant differences in their metastatic behaviour. The generation of new variants can be observed during the subsequent stages of tumor progression. Molecular analysis has identified changes in cell surface marker expression to be correlated with the metastatic potential of tumor cells. Examples of such markers are ICAM-1 and certain variants of CD44 (e.g. Mayer et al., *The Lancet* 342:1019, 1993). It has furthermore been found that the down regulation of the expression of desmosomal adhesion molecules such as E-cadherin and plakoglobin is involved in the detachment of tumor cells with metastatic potential from the coenobium (e.g. Birchmeier et al., in "Contrib. Oncol.", Rabes et al., eds., Karger, Basel 1992, 95). Subsequently, the expression of motility factors (e.g. scatter factor, transforming growth factors) and the secretion of proteases (cathepsin D, metalloproteinases, plasminogenic activator) favours the invasion of detached tumor cells through the basal membrane and the surrounding stroma of the primary organ (for a review see Birchmeier and Birchmeier, *Annu. Rev. Cell Biol.* 9:511, 1993). The survival of disseminated tumor cells in the secondary organ is affected by their

ability to escape immune surveillance, proliferate in response to growth factors produced by themselves or the surrounding stroma cells, and induce angiogenesis.

The above recited processes all contribute to the metastatic potential of the epithelial tumor cell of the invention and are therefore comprised by this term.

The term "immortalizing oncogene" relates to an oncogene with the potential to immortalize the epithelial tumor cell of the invention such that said tumor cell is thereafter capable of being continuously kept in cell culture. The term "immortalizing" therefore differs in the context of the present invention from the term "transforming" which denotes the capacity of a transforming principle to promote the transition of a normal cell into a dividing or non-dividing tumor cell. A transformed cell is characterized by loss or reduction of adherence dependent growth, loss of contact inhibition, and serum or growth factor independence.

Said immortalizing oncogene is expressed upon integration into a chromosome or another replicative genetic element such as stable mini-chromosomes in said epithelial tumor cell. In the case of EBV or papilloma virus, viral DNA can be found in the cell as episomes. Whereas said oncogene will usually employ the expression machinery of the immortalized cell, the regulatory sequences directing the expression of said oncogene may be both of intracellular or extracellular origin.

In a preferred embodiment of the present invention, said epithelial tumor cell is a disseminated tumor cell.

The term "disseminated tumor cell" refers to the fact that the tumor cell has already detached from the coenobium. It may e.g be in the blood stream or in the lymph system or be

settled in the final tissue of its migration such as bone marrow.

In a further preferred embodiment, the tumor cell of the invention is an autologous tumor cell.

In the context of the present invention, the term "autologous" means that the tumor cell is derived from the same organism into which it is, usually after genetic or other manipulation, reintroduced, e.g. for therapeutic purposes. Alternatively, said term denotes the case that the epithelial tumor cells of the invention are kept or tested in cell culture with cells derived from the same organism.

In a further preferred embodiment, the epithelial tumor cell of the invention is a human tumor cell.

In a further preferred embodiment of the invention, said epithelial tumor cell with metastatic potential is derived from bone marrow.

The term "derived from bone marrow" as used herein means that the tumor cell has infiltrated bone marrow and may be detected therein or isolated therefrom. Said term is not intended to mean that said tumor cell was before transformation a normal bone marrow cell.

The use of bone marrow as a preferred source of epithelial tumor cells with metastatic potential is based on the fact that the bone marrow compartment is a relatively easily accessible. Further, a number of epithelial tumors preferably invades the skeletal system (Zetter, New Engl. J. Med. 332:605, 1990). This may be explained by the finding that the last three steps of the cascade to metastasis development, namely the so-called arrest, the extravasation and the proliferation into the invaded tissue strongly

depend on tissue specific factors and are obviously favoured in the bone marrow.

A further preferred embodiment of the present invention relates to the above-characterized epithelial tumor cell wherein the immortalizing oncogene is a DNA encoding the early region of SV40 DNA and preferably the large T antigen of a replication deficient SV40 virus.

The term "DNA encoding the early region of SV40 DNA" is intended to mean any DNA molecule irrespective of the actual nucleotide sequence which encodes a large T antigen, small t antigen and/or the 17K T antigen, or a truncated form thereof, said T(t) antigen or truncated form thereof being capable of immortalizing the host cell. As a rule, the DNA encoding the large T antigen, small t antigen and/or 17K T antigen will be introduced into the tumor cell as a part of the complete or partial SV40 genome. The proper expression requires the SV40 enhancer at the 5' end and the poly A signal (AATAAA) at the 3' end (bp 2603) of the SV40 sequence.

The term "replication deficient SV40 virus" relates to the fact that the origin of replication of the SV40 DNA is not capable of being replicated or unwound. The replication deficiency can be caused by mutations in the origin of replication. Alternatively mutations in the SV40 early coding region can cause a replication defect.

In accordance with the present invention, the replication defect may most preferably be caused by (a) defect(s) such as (a) point mutation(s), (a) deletion(s), (an) insertion(s) etc. in the so-called "origin of replication" (ORI) of the SV40 genome.

In another most preferred embodiment of the invention, said replication defect is caused by one or more mutations,

insertions, deletions etc. in the antigen coding region. In this embodiment, which may or may not comprise a (defect) origin of replication, the defect e.g. in the large T coding region has the effect that immortalization of the host cell, but no virus DNA replication occurs, for example due to defects in the DNA-binding domain, helicase activity or other properties required for replication in SV40 large T antigen.

The replication deficiency in the SV40 DNA which is not necessary to carry out the invention but contributes to the overall safety of the system serves the purpose to prevent replication of the viral genome which could eventually lead to uncontrolled replication of the viral DNA and therewith to the destruction, and not the immortalization, of the hosting tumor cell.

In a further most preferred embodiment of the epithelial tumor cell of the present invention, said SV40 virus is non-infectious.

This most preferred embodiment is particularly suitable for the development of pharmaceutical compositions or tumor vaccines comprising the tumor cell of the invention. It is furthermore particularly suitable for any scientific or medical purpose where the non-infectiousness of the SV40 virus is desired or prescribed.

In order to achieve non-infectiousness of the SV40 virus, the viral core proteins may be inactivated such as by a deletion of a 546bp fragment in the DNA encoding VP1 and VP2 which is further illustrated in the examples.

In a further preferred embodiment of the present invention, at least one additional oncogene is integrated in the genome of the epithelial tumor cell.

Said additional oncogene may enhance the percentages of tumor cells which are immortalized upon the integration of the externally introduced immortalized oncogene.

In a most preferred embodiment of the present invention, said additional oncogene is ras, mutant WT1 (Wilms tumor), bcl-2, p53mut, myc, HER 2/neu, an HPV16 oncogene, an HPV18 oncogene or E1A. Depending on the host cell an additional oncogene could enhance or supplement the effect of SV40 T(t) antigens and related proteins.

A further preferred embodiment of the present invention relates to a tumor cell which has additionally integrated in its genome or another replicative genetic element at least one externally introduced gene encoding an immunostimulatory factor.

Modulation of immune response through the use of tumor cells genetically transduced with cDNA coding for cytokines (e.g. IL-2, IL4, IFN- α , IFN- γ) showed that it is possible to substantially improve tumor responses in animals; in some cases long term remissions of metastatic disease was achieved (reviewed in : Blankenstein T, Rowley DA; Schreiber H, 1991. Cytokines and Cancer: Experimental systems. Curr. Opin. Immunol. 3:694). More recently it was shown that most tumor cells lack important co-stimulatory factors such as proteins encoded by the B7 genes, which are required to induce an effective cellular immune response (reviewed in: Guinan EC, Gribben JG, Boussiotis VA, Freeman GJ, Nadler LM, Pivotal role of the B7:CD28 pathway in transplantation tolerance and tumor immunity. Blood 84:3261-3282, 1994). The transduction of these genes in tumor cells has resulted in a marked anti-tumor immune response in animal experiments. The expanding knowledge on the in vivo roles of cytokines and co-stimulatory factors should allow optimal combination therapies of cells and cytokines in the treatment of cancer in man.

In a most preferred embodiment, said immunostimulatory factor is B7 or a cytokine, e.g. IL-2, IL-4, IL-7, IFN- α or IFN- γ .

The present invention additionally relates to an antibody or fragment thereof or a derivative of said antibody or said fragment which specifically recognizes the epithelial tumor cell of the invention.

The term "specifically recognizes the epithelial tumor cell of the invention" means that the antibody is capable of distinguishing between the normal epithelial cell from which the tumor cell with metastatic potential is derived and a primary epithelial tumor cell on the one hand and the immortalized epithelial tumor cell with metastatic potential of the present invention on the other hand.

Said distinction will usually be caused by a differential expression of surface markers, as has been discussed herein above and is shown in Example 9 below.

The antibody of the invention may be a polyclonal, a monoclonal, a chimeric, a synthetic or a semisynthetic antibody. The generation of such antibodies including the design of immunogens, the immunization strategy as well as the isolation of the antibodies may follow well established protocols which are described e.g. in Harlow and Lane, Antibodies, A Laboratory Manual, CSH Press, Cold Spring Harbor 1988.

The term "fragment" of an antibody relates to any fragment capable of binding the tumor cell of the invention. Examples of said antibody fragments are Fab, F(ab)₂ or Fv fragments.

The term "derivative of said antibody or said fragment thereof" refers to a modified antibody or fragment of the

invention. This modification may be effected e.g. by genetic engineering or by chemical means. For example, the genetically engineered antibodies or fragments thereof comprise fusion proteins or single chain (VL or VH) derivatives. The chemically modified antibodies or fragments thereof also include chemical conjugates such as bispecific antibodies.

In a most preferred embodiment of the present invention, said antibody is a monoclonal antibody.

The present invention relates further to an in vitro process for the production of the tumor cell of the invention comprising the step of incorporating DNA comprising DNA encoding at least one immortalizing oncogene and optionally at least one gene encoding an immunostimulatory factor into a non-immortalized epithelial tumor cell with metastatic potential.

The process of the present invention for the first time allows the specific and unlimited expansion of tumor cells of epithelial origin with metastatic potential. In principle, the present invention can be applied to all cases where a selective expansion of a defined subpopulation of rare cells is wanted. Examples for such an application are the expansion of fetal erythroblasts from the maternal blood for the purpose of genetic diagnosis of inborn defects, and the expansion of hematopoiesis-supporting bone marrow stroma cells located in the close neighborhood of hematopoietic stem cell clusters, which could be used as feeder cells to enhance expansion of stem cells in vitro (e.g. for autologous stem cell transplantation of cancer patients undergoing high dose chemotherapy). The step of incorporation may comprise any suitable incorporation method. In a preferred embodiment of the method of the invention, said incorporation comprises microinjection or bombardement of the target cells with DNA-coated

microvesicles. The microinjection of cells has been well established in the art in the 1980's and need not be described here further. The microinjection step allows the specific introduction of the DNA encoding at least one oncogene capable of immortalizing the cells, preferably the large T antigen of SV40 into the desired tumor cells embedded e.g. in bone marrow tissue or kept in cell culture after enrichment. For a better identification, said cells may be tagged with a labelled antibody.

After the incorporation step, said cells may be cultivated and expanded and therefore establish a cell line with the characteristics of the present invention.

In a preferred embodiment of the process of the present invention, said process additionally comprises the step of carrying out a primary expansion of said non-immortalized epithelial tumor cells prior to the incorporation step.

This embodiment is particularly suitable for carrying out the process of the present invention because it allows the enrichment and selective proliferation of non-immortalized tumor cells prior to microinjection. Said expansion leads to an easier identification of the desired cells and, accordingly, significantly facilitates the incorporation, e.g. the microinjection process.

The expansion is most conveniently carried out under the following conditions: A standard culture medium (RPMI 1640) is supplemented with standard ingredients including 10 % fetal calf serum, 10 μ g/ml transferrin, 5 μ g/ml insulin, and 2 mM glutamine. According to the general rules of adherent cell culturing, the media are changed once to twice a week and fresh growth factors (as indicated below) are added to the cultures. At confluency the adherent cells (including the epithelial tumor cells) are removed by a standard

technique (incubation with trypsin/EDTA) and passaged into new culture flasks.

In a particularly preferred embodiment of the process of the present invention, said primary expansion comprises the step of culturing tissue or a body fluid comprising non-immortalized epithelial tumor cells in a suitable medium promoting the expansion of said tumor cells.

In a most preferred embodiment, said body fluid is bone marrow, blood, ascites or pleural exsudate.

In a further particularly preferred embodiment of the process of the present invention, the medium promoting the expansion of tumor cells comprises recombinant human (rh) EGF (epidermal growth factor) and/or rh bFGF (basic fibroblast growth factor).

In a further particularly preferred embodiment, the culturing step comprised in the method of the invention is carried out in extracellular matrix (ECM)-coated tissue flasks and/or at reduced oxygen concentrations of 5-10%. ECM provides important signals that inhibit apoptosis and support growth of epithelial tumor cells.

The present invention relates additionally to a pharmaceutical composition comprising the epithelial tumor cell of the invention and/or the antibody, derivative or fragment thereof of the invention, optionally in combination with a suitable pharmaceutical carrier.

The pharmaceutical composition of the present invention may be used for the prophylaxis of cancer. The pharmaceutical composition of the present invention may further be used for the treatment of cancer and/or metastasis of cancer.

The pharmaceutical composition containing the epithelial tumor cell of the invention may form the basis of an autologous or allogenic tumor cell vaccine, generated by suitable formulation and/or genetic modification of the cells such as transfection with immune stimulatory molecules and/or cytokines.

In a further preferred embodiment of the invention, the epithelial tumor cell or its genetic modification generated by transfection with immune stimulatory molecules and/or cytokines may be used to ex vivo stimulate a patient's immune cells isolated by known methods such as leukapheresis or isolation with magnetic beads. Reinfusion of such stimulated cells may generate an effective immune response to a patient's tumor.

In a further preferred embodiment the antibody, derivative or fragment thereof of the invention may be administered to a patient after surgical removal of a primary tumor in order to prevent tumor relapse.

The risk of tumor relapse for the individual patient will need to be precisely assessed and may vary depending on the presence of micrometastatic cells in bone marrow (Schlimok et al., Proc. Natl. Acad. Sci. 84:8672, 1987; Schlimok et al., Eur. J. Cancer 27:1461, 1991; Cote et al., J. Clin. Oncol. 9:1749, 1991; Diel et al., J. Clin. Oncol. 10:1534, 1992; Mansi et al., Br. J. Cancer 27:1552, 1991.; Harbeck et al., Br. J. Cancer 69:566, 1994, Lindemann et al., Lancet 340:685, 1992; Pantel et al., Cancer Res. 53:1027, 1993). A clear advantage of the minimal residual disease stage as target for a specific immunization approach is the accessibility of early disseminated cells, which frequently lodge in mesenchymal compartments, where potential effector cells abound (Riethmüller and Johnson, Curr. Op. Immunol. 4:647, 1992; Pantel and Riethmüller, Oncology Today 11:4, 1994).

Additionally, the present invention relates to a diagnostic composition comprising the epithelial tumor cell of the invention and/or the antibody of the invention.

The diagnostic composition of the present invention may be used for the detection of an epithelial tumor cell with metastatic potential in any suitable tissue such as blood and bone marrow.

Further, the present invention relates to the use of the epithelial tumor cell of the invention for the preparation of a medicament for the prophylaxis and/or treatment of cancer and/or metastasis of cancer.

The present invention also relates to the use of an antibody or derivative or fragment thereof of the invention for the preparation of a medicament for the prophylaxis and/or treatment of cancer and/or metastasis of cancer.

Finally, the present invention relates to the use of the epithelial tumor cell of the invention or the antibody or derivative or fragment thereof of the invention for the preparation of a tumor vaccine.

The figures show:

Fig. 1: Construction of transformation vector.

pUC12 is a member of a family of vectors with several common features. They contain a multiple cloning site in the lacZ-gene and carry an ampicillin resistance gene (Vieira, J. and Messing, J. , Gene, 19:259, 1982; Yanish-Perron, C., et al., Gene, 33:103, 1985). pUCIn wt was cloned by introducing the 4697 basepair SV40 genome fragment BamHI (bp 2533)-origin of replication- PstI (bp 1988) containing the origin of replication, in the

linker of pUC12. A 546 bp fragment in the late region of SV40 is deleted. The plasmid pUCIn1 contains 7359bp.

Fig. 2: Principle of Microinjection of SV 40 early gene DNA

The tumor cells were plated onto petri dishes (3.5 cm in diameter, Costar, Germany) one day before injection with the microinjector model 5171 (Eppendorf, Hamburg, Germany) mounted on an inverted IM35 microscope (Carl Zeiss, Oberkochen, Germany). After injection of 200 to 300 cells per plate, the cells were re-transferred into separate T25 culture flasks and cultured, as described above. An experienced worker needs about 30 minutes to inject 200 to 300 epithelial cells. Since non-injected cells normally die off after 4-8 weeks in culture, the SV40-transduced cells are selected by their persistent growth in culture.

Fig. 3: Expression of the SV40 large T antigen in microinjected epithelial cells from the bone marrow culture of a patient with bronchial carcinoma (magnification 125x).

Fig. 4: Primary in vitro expansion and immortalization of micrometastatic cancer cells.

(A-C) Primary cultures of bone marrow from three individual patients with prostate cancer who were staged as free of overt metastasis by conventional tumor staging procedures. At the indicated time points, the cultured cells were trypsinized and immunocytochemical screening for CK-positive tumor cells was performed. (D, E) SV 40 large T DNA-induced long term growth of micrometastatic tumor cells in culture. Primary bone marrow cultures established from a patient with either non-small

cell lung cancer (D) or prostatic cancer (E) were microinjected with SV 40 large T antigen DNA. Cells were trypsinized at the indicated time points and immunostained for expression of cytokeratin (\square) and large T antigen (\diamond)

Fig. 5: Detection of differential expression of surface markers on primary epithelial tumor cells and on immortalized epithelial tumor cells from bone marrow.

For further explanations see Example 9.

The Examples illustrate the invention.

Example 1

Construction of a transformation vector encoding the SV40 large T antigen and expression thereof.

In wt (Fig. 1) was cloned by integration of the SV40 genome fragments PstI/BstXI (2471bp) and BstXI/BamHI (2226bp) from pSVIn1 (Cohen et al., J. Virol 51:91, 1984) into pUC12. The fragments from pSVIn1 contain a disrupted origin generated by an insertion of one bp in the center of the 27 bp palindrome, thereby destroying the BglI site.

Example 2

Selection of patients and isolation of bone marrow aspirates.

After obtaining informed consent, bone marrow aspirates from 128 patients with primary carcinomas of the prostate, kidney, lung, breast and colorectum were investigated (Table 1). In addition, 17 patients without evidence for an epithelial malignancy were used as control group. This group

included patients with benign tumors and inflammatory diseases.

Bone marrow aspirates were obtained from both sides of the upper iliac crest through a aspiration needle. The volumes of all aspirates ranged from 2 to 10 ml (mean: 4 ml), yielding between 10^6 and 6×10^7 (mean: 2×10^7) mononuclear cells. After centrifugation through a Ficoll-Hypaque density gradient (Pharmacia, Germany; density 1.077 g/mol) at 900 g for 30 min, interface cells were cytocentrifuged on glass slides at 150 g for 5 min (8×10^4 cells per slide). Following overnight air drying, slides were either stained immediately or stored at -80°C prior to use with preservation of epithelial antigens for at least two years. From each aspirate five slides comprising 4×10^5 nucleated cells were examined (8×10^5 cells per patient), while one additional slide served as Ig isotype control.

Example 3

Immunocytochemistry

Two anti-cytokeratin (CK) mAbs (CK2 and A45-B/B3) were used for tumor cell detection in bone marrow cytospin preparations (Debus et al., EMBO J. 1:1982; Karsten et al., Eur. J. Cancer Clin. Oncol. 21:733, 1985; Schlimok et al., Proc. Natl. Acad. Sci. USA 84:8672, 1987; Pantel et al., J. Hematother. 3:165, 1994) (1) CK2 (IgG1; kindly provided by Dr. M. Osborn, Max-Planck-Institut Göttingen, and later obtained from Dr. H. Bodenmüller, Boehringer Mannheim, Tutzing, Germany) directed to the cytokeratin polypeptide No. 18 (CK18) (Debus et al., Am. J. Pathol., 1984; Debus et al., Am J. Pathol. 114:121, 1982). CK2 stains all "normal" (non malignant) cells of simple epithelia and tumors derived thereof as well as transitional cell carcinomas and a major fraction of squamous cell lung carcinomas (Pantel et al., Cancer. Res. 53:1027, 1993; Debus et al., Am. J. Pathol. 114:121, 1984; Debus et al., Embo. J. 1:1641, 1982; Hijazi

et al., J. Urol. 141:522, 1989). (2) mAb A45-B/B3 (IgG1; kindly provided by Dr. N. Karsten, Max-Delbrück Zentrum, Berlin, Germany), which detects a common epitope of a variety of cytokeratin components, including CK8, 18 and 19 (Karsten et al., Eur. J. Cancer Clin. Oncol. 21:733, 1985; Conrad et al., Biomed. Biochim. Acta 47:697, 1988). The mAbs were used at optimal concentrations, ranging from 2.5 - 4 mg/ml. Appropriate dilutions of unrelated mouse myeloma proteins served as IgG1 isotype control (MOPC21 from Sigma, Deisenhofen, Germany).

The antibody reaction was developed with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique combined with the Neufuchsin-method for visualizing antibody binding (Cordell et al., J. Histochem. Cytochem. 32:219, 1984). Briefly, after incubation with the primary antibody, a polyvalent rabbit anti-mouse Ig antiserum (Z259, Dako, Hamburg, Germany), and preformed complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase antibodies (D651, Dako, Hamburg, Germany), were used at the dilutions recommended by the manufacturer (Dakopatts, Hamburg, Germany). To allow a fast screening for mAb-positive cells on the slides, no counterstaining was performed.

Although this example employs two specific anti-cytokeratin antibodies, the person skilled in the art knows how to obtain monoclonal antibodies which serve the same purpose. The same holds true for the generation of antibodies with other specificities which would serve the same purpose as the antibodies described in the following examples.

For double staining a combined immunogold/enzymatic technique was used that has been successfully applied to the detection of histogenic and proliferation-associated markers on disseminated tumor cells (Riesenberg et al., Histochemistry 99:61, 1993). Briefly, cells were first incubated for 45 min. with mAb ER-PR8 (Gallee et al.,

Prostate 9:33, 1986) directed to prostate-specific antigen (PSA) (IgG1, Dakopatts, Hamburg, Germany). After a thorough wash with phosphate-buffered saline, gold-conjugated goat anti-mouse immunoglobulins were incubated for 45 min. Subsequently, the slides were washed and exposed to 2% glutaraldehyde diluted in phosphate-buffered saline for 5 min. The following immunoenzymatic step was similar to the single labelling method described above, except for the use of biotinylated mAb CK2, which was developed with preformed complexes of streptavidin and alkaline phosphatase. After rinsing in distilled water for at least 15 min, the freshly prepared silver enhancement mixture (Amersham, Braunschweig, Germany) was applied for about 20 min and the reaction was monitored every 5 min by placing the slides under the microscope. To abrogate the enhancement reaction, the slides were rinsed in distilled water. All slides were examined by two independent observers in a double-blinded fashion.

Example 4

Cell culture of epithelial tumor cells

Between 1 to 6×10^7 MNC were plated onto extracellular matrix-coated culture flasks (Paesel & Lorei, Frankfurt, Germany) and incubated at 5-10 % oxygen (Heraeus incubator B 5061 EK/O2, München, Germany). The culture medium contained RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mg/ml transferrin, 5 mg/ml insulin, 2 mM glutamine, 10 ng/ml recombinant human epidermal growth factor (Boehringer Mannheim, Mannheim, Germany) and 10 ng/ml recombinant human basic fibroblast growth factor (PBH, Hannover, Germany).

In the first step of an in vitro expansion of the epithelial cancer cells, a 3 - 5 ml inoculum of bone marrow aspirate was incubated under culture conditions that favoured the outgrowth of epithelial cell colonies in tissue culture flasks coated with extracellular matrix proteins. By trial

and error an optimal medium composition was found that contained equal amounts of basic fibroblastic growth factor and epidermal growth factor as well as the other ingredients as indicated above. As demonstrated in Table 1, bone marrow cultures were initiated from 128 patients with resected small primary cancers of breast, large bowel, lung, kidney and prostate (see Example 2). 42 of these patients exhibited CK+ cells in fresh marrow aspirates at an average frequency of 1 to 10 cells per 8×10^5 bone marrow cells, whereas only 8 patients had 10 or more CK+ cells (Table 1).

Under the described culture conditions, first clusters of epithelial cells appeared within 2 weeks (Fig. 1B). Significant numbers of CK+ cells ($> 10^3$) were observed in 55 cases (43.0 %) after 4 to 6 weeks of culture (Table 2). Depending on the type of the primary tumor, the absolute number of CK+ cells usually increased by 2 to 4 logs (> 13 cell divisions) during this time. The median doubling times varied from 2 to 13 days. The concentration of CK+ cells increased with time since the hematopoietic bone marrow cells waned and died off, as demonstrated by sequential staining for the common leukocyte antigen CD 45, using mAb T9/33 (Trowbridge, J. Exp. Med. 148:313, 1978). The growth rates observed in vitro do not necessarily reflect the in vivo growth patterns. In bone-seeking tumors like cancer of the prostate, lung and breast culturing of bone marrow did not lead more frequently to outgrowth of epithelial cells than in colorectal cancer (Table 2), in whom manifest metastasis of skeleton is rare (Weiss L., Grundmann E. Thorhorst J. et al., Haematogenous metastatic patterns in colonic carcinoma: an analysis of 1541 necropsies. J. Pathol 150:195-203, 1986).

It is noteworthy that the number of CK+ tumor cells detected in a particular bone marrow sample did not correlate closely to the rate of expansion achieved in the subsequent culture. Twenty out of the 55 samples which gave rise to a significant expansion ($> 10^3$ CK+ cells per culture) presented with no detectable tumor cells per 8×10^5 MNC

analysed. With regard to the total number of MNC plated per culture ($1-6 \times 10^7$), it can be assumed that most of these cultures were performed with epithelial tumor cells present under limiting dilution conditions. On the other hand, CK+ cells detected in 15 of the 50 CK+ marrow samples failed to grow in vitro, indicating a reduced plating efficiency of these cells. To enhance the outgrowth of micrometastatic cells from these patients, a larger volume of bone marrow cells might be needed. Taken together, although micrometastatic cancer cells are presumably selected in vivo for their disseminative capability, the in vitro growth potential of these cells remains heterogeneous.

Epithelial cells, as defined by CK-staining, were not detected in bone marrow cultures from 17 patients without evidence of malignant epithelial tumors (Table 2). Also, the primary aspirates of their bone marrow were negative for CK+ cells. Therefore it appears unlikely that ectopic expression of cytokeratins (Traweek et al., Am. J. Pathol 142:1111, 1993; Galmiche et al., Blood 82:66, 1993) is induced by specific culture conditions described herein. The epithelial origin of CK+ cells in culture was further supported by the expression of PSA in the case of patients with prostate cancer (Figure 1F), as well as by the absence of the CD45 leukocyte common antigen and the mesenchymal intermediate filament vimentin, a marker for bone marrow stromal cells (Galmiche et al., Blood 82:66, 1993). Further evidence that the expanded epithelial cells are tumor-derived is provided by the presence of a mutated (codon 12) Ki-ras oncogene, detected by PCR (Kahn et al., Oncogene 6:1079, 1991), in marrow of five patients with CK+ cells. In addition, the G250 antigen associated with renal cell cancer (Oosterwijk et al., Int. J. Cancer 38:489, 1986) was found in all of the six cultures analyzed from patients with renal cell cancer.

Example 5

Microinjection of epithelial tumor cells

The expansion of epithelial tumor cells unambiguously showed that the life span of these CK+ cells in culture was limited. Although the longest survivors were viable for more than 200 days (Figure 2A), proliferation of CK+ cells ceased in more than 90% of the cultures after about 40 days, when the cells began to die off rapidly (Figure 2B,C). In order to rescue the expanded cells, it was tried to immortalize them with a well-defined oncogene. Among the various potential candidates with proven transforming capacity, the SV40 large T antigen appeared to be a most suitable one because of its well-established ability to immortalize or extend the life span of normal epithelial cells from various organs (Bartek et al., Proc. Natl. Acad. Sci. USA 88:3520, 1991; Berthon et al., Int. J. Cancer, 1992; Cussenot et al., J. Urol. 146:881, 1991; Bartek et al., Int. J. Cancer 45:1105, 1990; Fauth et al., Renal Physiol. Biochem. 14:128, 1991). For the purpose of the present invention, the observation was of particular interest that the SV40 large T antigen-transduced cells retained the differentiated phenotype of the original cells (Bartek et al., Proc. Natl. Acad. Sci. USA 88:3520, 1991; Berthon et al., Int. J. Cancer 52:92, 1992; Cussenot et al., J. Urol. 146:881, 1991).

Microinjection was chosen as an efficient route of gene transfer (Bartek et al., Int. J. Cancer 45:1105, 1990; Fauth et al., Renal Physiol. Biochem. 14:128, 1991) that allowed the selective transduction of epithelial tumor cells in the established primary marrow cultures.

The tumor cells were plated onto petri dishes (3.5 cm in diameter, Costar, Germany) one day before injection with the microinjector model 5171 (Eppendorf, Hamburg, Germany) mounted on an inverted IM35 microscope (Carl Zeiss, Oberkochen, Germany). After injection of 200 to 300 cells

per plate, the cells were re-transferred into separate T25 culture flasks and cultured, as described above. At different time points the adherent cells were detached by trypsin-EDTA treatment and immunostained using the APAAP method. Expression of histogenetic markers was assessed by staining with the anti-CK mAbs CK2 and A45-B/B3 as well as anti-PSA mAb ER-PR8, mAb G250 to a renal cell carcinoma-specific antigen (Osterwijk et al., Int. J. Cancer 38:489, 1986), CO17-1A to the 17-1A antigen (Gottlinger et al., Int. J. Cancer 38:47, 1986), and BR55-2 to the Lewis 6 blood group precursor antigen (Blaszczyk, 1987). Expression of the SV40 large T antigen was demonstrated by labelling with mAbs 220, 419, and 416 (Harlow et al., J. Virol. 39:861, 1981). As demonstrated in Table 3, microinjection of said 200 to 300 epithelial tumor cells per culture with linearised SV40 large T antigen DNA induced the continuous growth of epithelial cells in cultures from 37 of 75 cancer patients. The growth kinetics was studied in all samples and two representative experiments are shown in Figure 3. An exponential increase in T antigen-positive cells was followed by a rapid increase in the number of CK+ cells. The transduced cells grow to confluency within 3 to 5 weeks after microinjection (Figure 1D). T antigen expression was observed after more than 150 days in culture, suggesting the stable integration of the injected DNA (Fig. 1E and Fig. 3).

Example 6

Transplantation of microinjected epithelial tumor cells into SCID mice

Subcutaneous transplantation of one of the immortalized micrometastatic lung cancer cell lines into immunodeficient SCID mice resulted in the development of local tumor growth and micrometastatic bone marrow infiltration at a frequency of 10^{-4} . Such minimal marrow infiltration was also observed with another line of immortalized prostate cancer cells when

a subcutaneous tumor was not detectable (data not shown). This finding suggests that micrometastatic cells propagated from bone marrow aspirates still inherit a particular organspecific homing affinity in SCID-mice. The reconstruction of these mice with autologous immune effector cells might therefore allow an in depth investigation of the in vivo immune response against minimal residual cancer. The development of such an animal model is of utmost importance for the preclinical testing of genetically engineered tumor cell vaccines directed against minimal residual cancer.

Example 7

Phenotypic analysis of epithelial differentiation antigens in the immortalized tumor cells

A detailed phenotypic analysis of epithelial differentiation antigens in the expanded CK+ cells is summarized in Table 3. According to the origin of the individual tumor differentiation markers such as prostate-specific antigen (Fig. 1F), intestinal-specific annexin, prolactin-inducible protein and G250 were found in SV40 T antigen immortalized CK+ cells. The experimental details are as follows:

Expression of histogenetic markers at the mRNA level was determined by reverse transcriptase PCR (RT-PCR) analysis. Total RNA of about 10^6 cells of each evaluated culture was purified with the guanidine thiocyanate method. 2 ml total RNA was reverse transcribed into cDNA using specific primers for intestine-specific annexin (ACTATGCGAATCAACGTC), prostate-specific antigen (TGACGTGATACCTTGAAGCA) and gross-cystic disease-fluid-protein-15/prolactin-inducible-protein (GTGTGGCAAACAGACAGG) (Schulz et al., Nucleic. Acids. Res. 16:6226, 1988; Wice and Gordon, J. Cell Biol. 116:405, 1992; Murphy et al., J. Biol. Chem. 262:15236, 1987). The following PCR primers were used for amplification of marker-specific cDNA:

PSA: 5'CTTGTAGCCTCTCGTGGCAG, 3'GACCTTCATAGCATCCGTGAG;
ISA: 5'CATTGAGTTCCTGTGCACGA, 3'GACTTGTACAGACGTTTCAGC;
PIP: 5'CAAAGCTCAGGACAACACTCG, 3'CAGCATCATCAGGGCAGATG.

The specificity of the amplification products was demonstrated by restriction enzyme analysis.

The results and above observations suggest that integration of the SV40 DNA and expression of large T antigen did not substantially change the expression pattern of the studied differentiation markers.

Recent analyses of a number of T antigen mutants have clearly shown that the specific DNA binding properties of T antigen are not essential for immortalization (Fanning and Knippers, *Annu. Rev. Biochem.* 61:55, 1992; Dobbelstein et al., *Oncogene* 7:837, 1992). However, SV40 large T antigen interacts specifically with the products of two known tumor suppressor genes, the p300 or related functions (Yaciuk et al., *Mol. Cell. Biol.* 11:2116, 1991) and with retinoblastoma susceptibility (RB) gene and the p53 gene, thereby neutralizing the growth-arresting properties of both proteins (Fanning and Knippers, *Annu. Rev. Biochem.* 61:55, 1992; Dobbelstein et al., *Oncogene* 7:837, 1992). In the present study, the level of p53 protein detectable by immunocytochemistry increased in immortalized micrometastatic cells, suggesting that p53 protein interacted with T antigen in these cells, which in turn prolonged the half-life of p53. Since the p53 protein is thought to be frequently defective or absent in metastatic epithelial tumor cells, it is surprising that this interaction is required to obtain the described immortalisation of micrometastatic carcinoma cells. However, extensive comparative evaluations of immortalized and parental tumor cells and studies with T antigen mutants need to be performed to determine the molecular basis for the observed immortalization. The usefulness of SV40 T antigen-transduced cells as vaccines is supported by the observation that the expression of molecules relevant to an efficient

immune response, such as MHC class I antigens and ICAM-1, is not downregulated in the genome of micrometastatic cells expressing T antigen (data not shown), which is consistent with the results of previous investigations on various other types of human and rodent cells (Galy et al., Thymus 22:13, 1993; Vidal et al., J. Immunol. Methods 166:63, 1993; Tanaka et al., Eur. J. Immunol. 23:2614, 1993). The method established here appears to be a feasible way to generate large quantities of cells that are derived from the earliest metastasizing cells and that apparently have conserved the phenotype of the residual tumor cells present in the patient. The availability of such cells opens a new avenue to an in-depth molecular analysis of cancer micrometastasis and may, e.g., be useful as a novel source for autologous tumor cell vaccines. The great advantage of this source is that it could be applied in the critical stage of minimal residual cancer, when the tumor load is minimal and the immune system is still intact (Riethmüller, G. and Johnson J.P., Current Opinion in Immunology 4 (1992), 647-655).

Example 8

In vitro generation of an autologous T cell response to micrometastatic carcinoma cells propagated from bone marrow.

For specific immunization against minimal residual cancer, micrometastatic cancer cells themselves are the ideal target candidates. The evaluation of the immune response against these tumor cells is, however, hampered by their extremely low frequency (e.g. 10^{-5} - 10^{-6} in bone marrow). In the present study, culture conditions allowing a more than 10,000-fold transient expansion of early disseminated epithelial tumor cells present in bone marrow were established. Expanded cells were immortalized by selective microinjection with SV 40 large tumor antigen DNA without gross changes in the expression pattern of epithelial antigens. In one of the cell lines (PC-MM-1) established

from bone marrow of a prostate cancer patient without distant overt metastasis (stage M₀), the gene encoding for the co-stimulatory molecule B7 was transduced in a retroviral vector. Immunostaining of these cells revealed the following phenotype: HLA-A2⁺/A3⁺, HLA-Bw4⁻/Bw6⁺ and ICAM1⁺. Incubation with 10U/ml IFN- γ for 72 h increased the expression of these molecules except for Bw4. Autologous PBL were in vitro stimulated on an irradiated monolayer of the B7 transfected tumor cells in medium supplemented with 10 U/ml IL-2 and 40 U/ml IL-4. The tumor cell monolayer was completely killed by the PBLs after three days in culture. Restimulation was performed by renewing the irradiated target monolayer once a week. After four rounds of stimulation the PBL population consisted of 75% - 85% CD3⁺CD8⁺T-cells. In a ⁵¹Cr-release assay with an effector to target ratio of 50:1 we observed a lysis of 15% to 20% B7-transfected PC-MM-1. The observation that IFN- γ pre-treated LNCap prostate cancer cells were also lysed at a similar rate suggests that the observed T-cell response might be prostate associated. In conclusion, transduction of the B7 gene into micrometastatic tumor cells might be an efficient way to enhance the cellular immune response against these cells and to generate large quantities of specific cytotoxic effector cells from peripheral blood samples.

Example 9

Detection of differential expression of surface markers on primary epithelial tumor cells and on immortalized epithelial tumor cells from bone marrow

All experimental procedures used in this example are standard procedures. mRNA was prepared from tumor cells (renal cell carcinoma) and from CK⁺ cells from bone marrow and reverse transcribed into cDNA using oligo-dT-priming. "Hot" PCR, i.e. PCR with radioactively labeled material was carried out with random and oligo-dT-primers. The products

of said PCR were analysed using urea-polyacrylamide electrophoresis. Fig. 5 shows the results of two such experiments. In Fig. 5a tumor specific bands and in Fig. 5b bands specific for CK+ cells are clearly visible. Next, the bands that were differentially amplified were isolated, cloned and sequenced. Full length cDNA was obtained using conventional DNA libraries obtained from the above cells. Full-length cDNAs were cloned in E.coli, followed by recombinant protein expression in E.coli. The recombinant proteins were used to generate specific polyclonal antisera in mice. Specificity of the sera tumor cells from bone marrow was confirmed using native tumor probes. Finally, monoclonal antibodies were generated against the specific proteins.

TABLES

Table 1. Frequency of CK-positive micrometastatic tumour cells in bone marrow. For detection of tumour cells, mononuclear cells (MNC) isolated from bone marrow aspirates were immunostained, using the APAAP-staining technique with anti-cytokeratin mAbs CK2 and A45-B/B3.

Tumour type	Number of patients analysed	Frequency of CK-positive cells in bone marrow* (number of patients with n CK ⁺ cells per 8×10^5 MNC)		
		<1	1 - 5	6 - 10
Prostate cancer	22	8	11	1
Renal cell cancer	12	9	3	0
Lung cancer	14	9	4	1
Breast cancer	41	28	8	2
Colorectal cancer	39	24	11	1
Total :	128	78	37	5

* based on the analysis of 8×10^5 MNC isolated through density gradient centrifugation from bone marrow aspirates of the upper iliac crest and/or sternum.

Table 2. Expansion and enrichment of CK-positive micrometastatic tumour cells. Between 1 to 6×10^7 MNC obtained from bone marrow aspirates were cultured over 4 to 8 weeks.

Tumour type	Number of patients	Number of cultures with $>10^3$ CK+ cells	Maximum Expansion (x n CK+ cells) [#]	Maximum Enrichment (x conc. CK+ cells) [#]
Cancer, total	128	55 (43.0%)	11000	1.1×10^6
Prostate cancer	22	12 (54.5%)	11000	1×10^5
Renal cell cancer	12	6 (50.0%)	690	8.8×10^3
Lung cancer	14	8 (57.1%)	2830	1.1×10^6
Breast cancer	41	13 (31.7%)	4410	1.6×10^5
Colorectal cancer	39	16 (41.0%)	2440	2.6×10^4
Control group*	17	0	0	0

[#] as defined by sequential immunostaining with anti-CK mAbs CK2 and A45-B/B3 was performed before and after primary culture.

* patients with benign tumours and inflammatory diseases

Table 3. SV40 large T antigen-induced long term propagation of micrometastatic tumour cells.

Tumour type	Number of patients	Number of passages (mean value)	Epithelial markers (Number of positive cultures)
Prostate cancer	22	2 - 104 (15)	Cytokeratin (22) Prostate-specific antigen (8)
Renal cell cancer	7	2 - 6 (4)	Cytokeratin (7) G250 (4)
Breast cancer	3	4 - 16 (10)	Cytokeratin (3) Prolactin-inducible protein (2)
Lung Cancer	3	3 - 85 (36)	Cytokeratin (3) 17-1A/55-2 antigens (2)
Colorectal cancer	2	9, 12	Cytokeratin (2) 17-1A/55-2 antigens (1) Intestinal-specific annexin (1)

200 - 300 tumour cells of each patient were microinjected after primary in vitro expansion, as described hereinabove. Protein expression was assessed by immunocytochemistry with anti-CK mAbs CK2 and A45-B/B3, ER-PR8 to PSA, G250 to a renal cell carcinoma-specific antigen and 17-1A/SDZ ABL 364 to the 17-1A/BR55-2 antigens, mRNA expression of PSA, prolactin-inducible protein, and intestinal-specific annexin was determined by RT-PCR analysis.

CLAIMS

1. An epithelial tumor cell with metastatic potential which has integrated in its genome or another replicative genetic element an externally introduced immortalizing oncogene which is expressed in said cell.
2. The epithelial tumor cell according to claim 1 which is a disseminated tumor cell.
3. The epithelial tumor cell according to claim 1 or 2 which is an autologous tumor cell.
4. The epithelial tumor cell according to any one of claims 1 to 3 which is a human tumor cell.
5. The epithelial tumor cell according to any one of claims 1 to 4 which is derived from bone marrow.
6. The epithelial tumor cell according to any one of claims 1 to 5, wherein said immortalizing oncogene is the DNA encoding the early region of SV40 DNA and preferably the large T antigen of a replication deficient SV40 virus.
7. The epithelial tumor cell according to claim 6, wherein said replication deficiency is caused by (a) defect(s) in the origin of replication and/or (a) defect(s) in the antigen coding region.
8. The epithelial tumor cell according to claim 6 or 7, wherein said SV40 virus is non-infectious.
9. The epithelial tumor cell according to any one of claims 1 to 8 which has integrated in its genome at least one additional oncogene.

10. The epithelial tumor cell according to claim 9, wherein said additional oncogene is ras, mutant WT1, bcl-2, p53mut, myc, HER 2/neu, an HPV16 oncogene, an HPV18 oncogene or E1A.
11. The epithelial tumor cell according to any one of claims 1 to 10, which has additionally integrated in its genome or another replicative genetic element at least one externally introduced gene encoding an immunostimulatory factor.
12. The epithelial tumor cell according to claim 11, wherein said immunostimulatory factor is B7 or a cytokine, preferably IL-2, IL-4, IL-7, IFN- α or IFN- γ .
13. An antibody or fragment thereof or a derivative of said antibody or said fragment thereof which specifically recognizes the tumor cell according to any one of claims 1 to 12.
14. The antibody or fragment thereof according to claim 13 which is monoclonal antibody or fragment thereof.
15. The derivative or fragment according to claim 13 which is a fusion protein or a chemical conjugate, preferably a bispecific antibody.
16. An in vitro process for the production of the tumor cell according to any one of claims 1 to 12 comprising the step of incorporating DNA comprising DNA encoding at least one immortalizing oncogene and optionally at least one gene encoding an immunostimulatory factor into a non-immortalized epithelial tumor cell with metastatic potential.

17. The process according to claim 16, wherein said step of incorporating DNA comprises microinjection or bombardment with DNA-coated microparticles.
18. The process according to claim 16 or 17, additionally comprising the step of carrying out a primary expansion of said non-immortalized epithelial tumor cells prior to the microinjection or bombardment step.
19. The process according to claim 18, wherein said primary expansion comprises the step of culturing tissue or a body fluid comprising non-immortalized epithelial tumor cells in a suitable medium promoting the expansion of said tumor cells.
20. The process according to claim 19, wherein said body fluid is bone marrow, blood, ascites or pleural exsudate.
21. The process according to claim 19 or 20, wherein said medium comprises EGF and/or bFGF, preferably rh EGF and rhb FGF.
22. The process according to any one of claims 19 to 21, wherein said culturing step is carried out in ECM-coated tissue flasks and/or at reduced oxygen concentrations of 5-10%.
23. A pharmaceutical composition comprising the epithelial tumor cell according to any one of claims 1 to 12 and/or the antibody or derivative or fragment thereof of any one of claims 13 to 15, optionally in combination with a suitable pharmaceutical carrier.

24. A diagnostic composition comprising the epithelial tumor cell according to any one of claims 1 to 12 and/or the antibody or derivative or fragment thereof of any one of claims 13 to 15.
25. Use of the epithelial tumor cell according to any one of claims 1 to 12 for the preparation of a medicament for the prophylaxis and/or treatment of cancer and/or metastasis of cancer.
26. Use of the antibody or derivative or fragment thereof according to any one of claims 13 to 15 for the preparation of a medicament for the prophylaxis and/or treatment of cancer and/or metastasis of cancer.
27. Use of the epithelial tumor cell according to any one of claims 1 to 12 or the antibody or derivative or fragment thereof of any one of claims 13 to 15 for the preparation of a tumor vaccine.
28. Use of the epithelial tumor cell according to any one of claims 1 to 12 for the ex vivo stimulation of a patient's immune cells.

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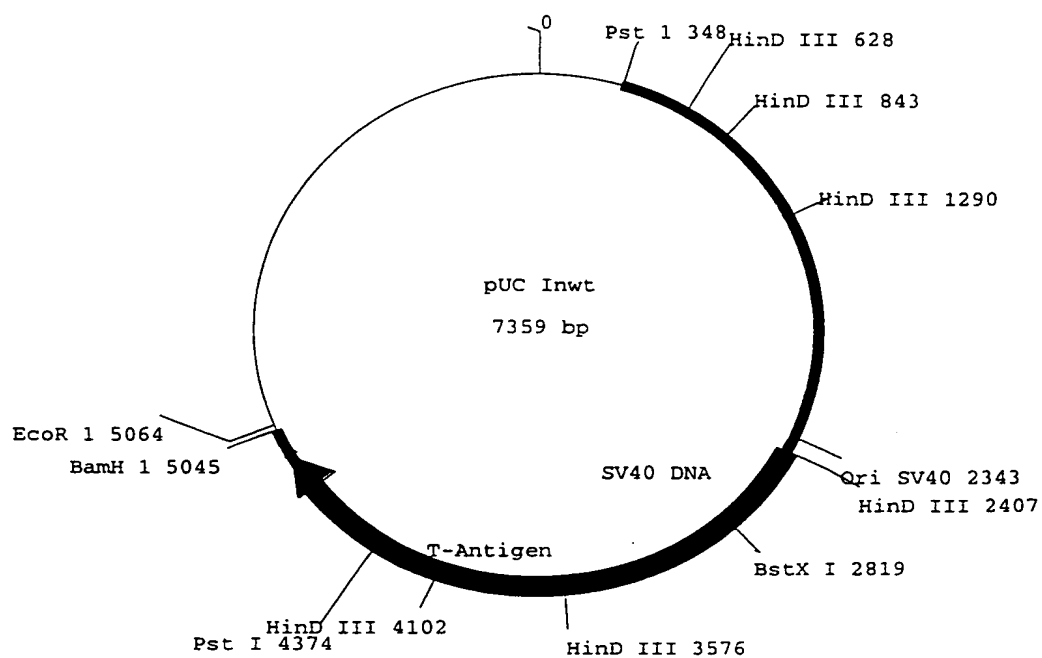


FIG. 1

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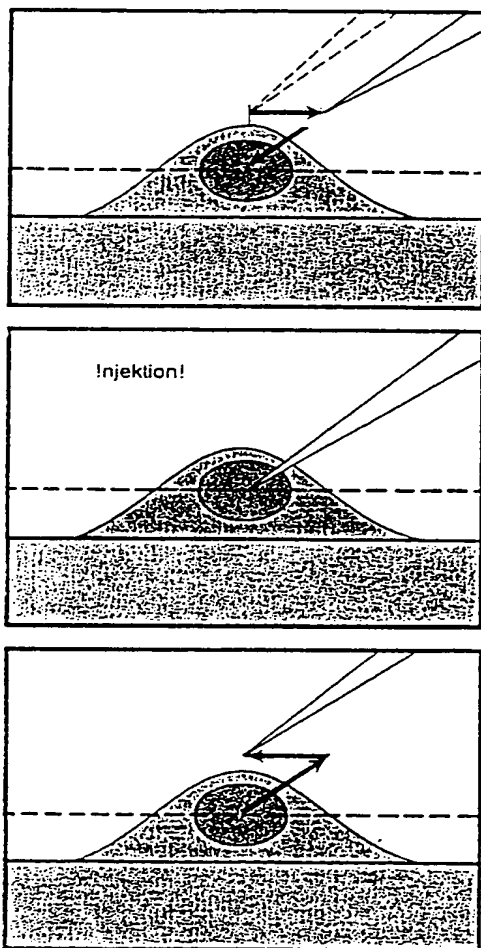


FIG. 2

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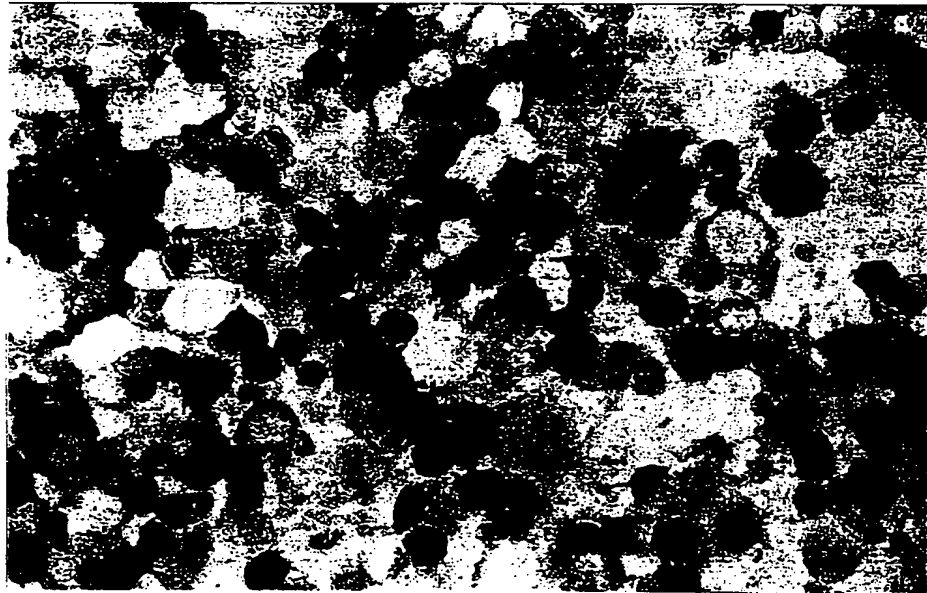


FIG. 3

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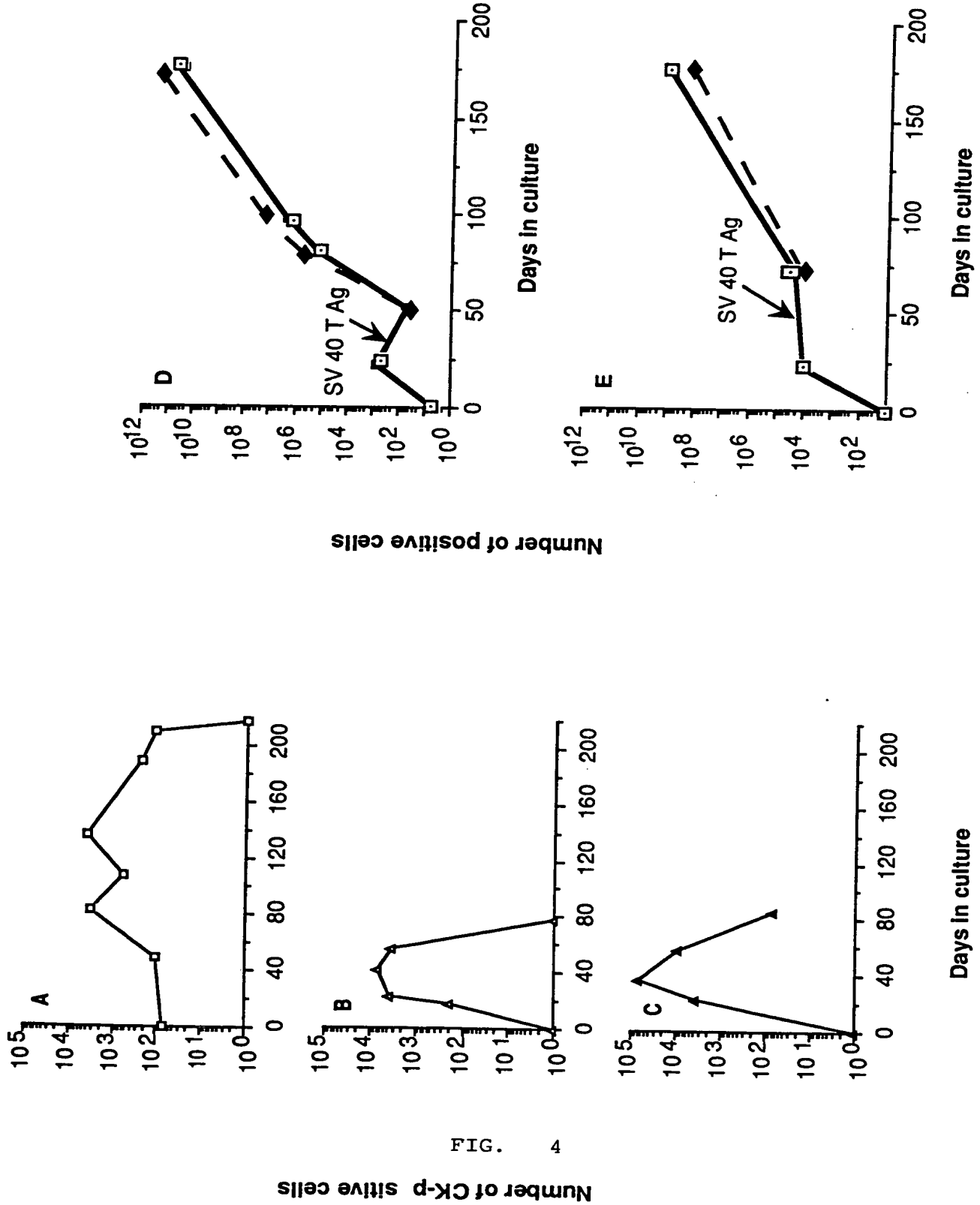


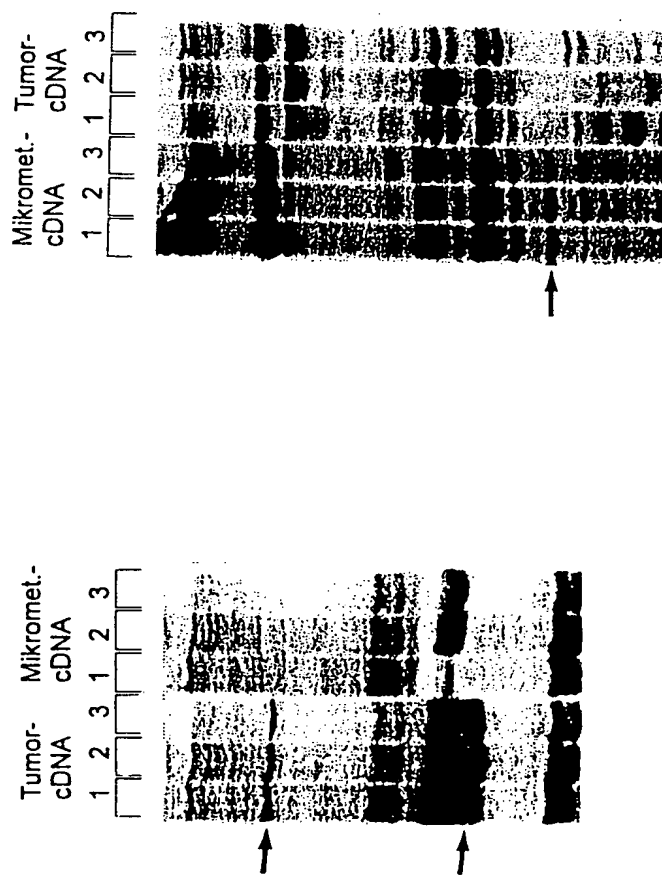
FIG. 4

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Fig. 5

Differential Display: Specific PCR-Products

- a) G1: tumor specific bands
- b) C2: bands specific for immortalized epithelial tumor cells from bone marrow



INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/EP 96/02747

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N5/10 C07K16/30 A61K39/00 A61K39/395 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER RESEARCH, vol. 49, 1989, pages 4615-4621, XP000611733 URA ET AL: "EXPRESSSION OF TYPE IV COLLAGENASE AND PROCOLLAGEN GENES AND ITS CORRELATION WITH THE TUMORIGENIC, INVASIVE, AND METASTATIC ABILITIES OF ONCOGENE-TRANSFORMED HUMAN BRONCHIAL EPITHELIAL CELLS" see the whole document ---	1-28
X	JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 81, 1989, pages 587-594, XP000611732 BONFIL ET AL: "INVASIVE AND METASTATIC POTENTIAL OF A V-HA-RAS-TRANSFORMED HUMAN BRONCHIAL EPITHELIAL CELL LINE" see the whole document ---	1-28
	- / - -	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

4 December 1996

Date of mailing of the international search report

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

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INTER IONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 85, 1993, pages 1419-1424, XP000611728 PANTEL ET AL: "DIFFERENTIAL EXPRESSION OF PROLIFERATION-ASSOCIATED MOLECULES IN INDIVIDUAL MICROMETASTATIC CARCINOMA CELLS" see the whole document ---	1-28
A	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 87064487, GARCIA ET AL: "ESTABLISHMENT OF TWO RABBIT MAMMARY EPITHELIAL CELL LINES WITH DISTINCT ONCOGENIC POTENTIAL AND DIFFERENTIATED PHENOTYPE AFTER MICROINJECTION OF TRANSFORMING GENES" XP002020222 & MOLECULAR AND CELLULAR BIOLOGY, (1986 JUN) 6 (6) 1974-82 see abstract ---	
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International Application No

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